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Enzymatic synthesis of novel quercetin sialyllactoside derivatives

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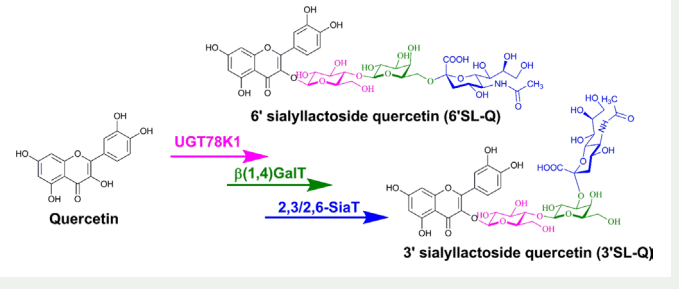
ABSTRACT

Quercetin and its derivatives are important flavonols that show diverse biological activity, such as antioxidant, anticarcinogenic, anti-inflammatory, and antiviral activities. Adding different substituents to quercetin may change the biochemical activity and bioavailability of molecules, when compared to the aglycone. Here, we have synthesised two novel derivatives of quercetin, quercetin-3-*O*-*β*-D-glucopyranosyl, 4″-*O*-D-galactopyranosyl 3‴-*O*-*α*-*N*-acetyl neuraminic acid i.e. 3′-sialyllactosyl quercetin (3′SL-Q) and quercetin-3-*O*-*β*-D-glucopyranosyl, 4″-*O*-*β*-D-galactopyranosyl 6‴-*O*-*α*-*N*-acetyl neuraminic acid i.e. 6′-sialyllactosyl quercetin (6′SL-Q) with the use of glycosyltransferases and sialyltransferases enzymes. These derivatives of quercetin were characterised by high-resolution quadrupole-time-of-flight electrospray ionisation mass spectrometry (HR-QTOF-ESI/MS) and ^{1}H and ^{13}C nuclear magnetic resonance (NMR) analyses.



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1. Introduction

Quercetin is an important constituent of the flavonoid family and is found in many fruits and vegetables, as well as red wine, and tea (Erlund 2004). Quercetin has been reported to exhibit various pharmacological activities such as antioxidative (Murota and Terao 2003),

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anti-inflammatory (Kleemann et al. 2011), anticarcinogenic (Yang et al. 2001), antiangiogenic (Tan et al. 2003), and anti-melanoma (Cao et al. 2014) effects. Different substituents attached to quercetin change the biochemical activity and bioavailability of molecules when compared to the aglycone (Materska 2008). The conjugation of quercetin with specific sugar motifs is an effective strategy to improve the biological activity of quercetin *in vivo* (Makino et al. 2013). There was 3- to 40-fold enhancement in the anticancer activity of geldanamycin antibiotic, which was conjugated with galactose and lactose analogues when they were incubated with *β*-galactosidase in the cells (Cheng et al. 2005). Sialic acids (SAs) are 9-carbon monosaccharides directly involved in many important biological and pathological processes (Chen and Varki 2010). Several naturally occurring SA forms have been found in sialic acid-containing glycosphingolipids, namely gangliosides (GM1 and GM2). Since the biology of SA is closely linked to human health, sialic acids have considerable significance in terms of human health, disease prevention, drug discovery, and immunity modulation (Bagriaçik and Miller 1999; Varki and Gagneux 2012; Bisel et al. 2014). SAs being exposed to the terminal glycan chain have become a potential target for glycan remodeling for the synthesis and design of novel medicines and vaccines (Byrne et al. 2007). It has been reported that quercetin 7-*O*-sialic acid show better anti-oxidation, anti-inflammation, cholesterol efflux promotion, and protection of biomolecules from desialylation than quercetin (Tian et al. 2017). Recently, two sialylated human milk oligosaccharides 3′-sialyllactose and 6′-sialyllactose have shown an ability to neutralize different avian influenza viruses (Pandey et al. 2018).

In the current experiment, a regiospecific glycosyltransferases (UGT78K1) from *Glycine max* and *β*-1,4-galactosyltransferase [*β*(1,4)GalT] were used for the synthesis of quercetin-3-*O*-*β*-D-glucopyranosyl, 4′′-*O*-*β*-D-galactoside i.e. quercetin-3-*O*-*β*-D-lactoside (QL) from quercetin. In addition, an *α*-*N*-acetyl neuraminic acid group was conjugated at 3′′′ and 6′′′-OH group of galactose of quercetin-3-*O*-*β*-D-lactoside to synthesize quercetin-3-*O*-*β*-D-glucopyranosyl, 4′′-*O*-*β*-D-galactopyranosyl 3′′′-*O*-*α*-*N*-acetyl neuraminic acid i.e. 3′-sialyllactosyl quercetin (3′SL-Q) and quercetin-3-*O*-*β*-D-glucopyranosyl, 4′′-*O*-*β*-D-galactopyranosyl 6′′′-*O*-*α*-*N*-acetyl neuraminic acid i.e. 6′-sialyllactosyl quercetin (6′SL-Q) (Figure 1), respectively by using two different sialyltransferases. Sialyllactose conjugated flavonoids have not been reported yet.

2. Results and discussion

2.1. In vitro *enzyme-catalysed quercetin-3-O-β-D-glucoside synthesis*

Quercetin-3-*O*-*β*-D-glucoside (QG) was synthesised from the one-pot reaction as described in materials and methods. A *Glycine max* glucosyltransferase UGT78K1 enzyme catalysed more than 90% conversion of the 8 mM of quercetin to QG, which was purified and used as a substrate for successive enzymatic reactions, to generate other derivatives. The high performance liquid chromatography photodiode array (HPLC-PDA) of the reaction mixture showed a prominent new single peak at retention time of 8.6 min, while the peak of quercetin was observed at 11.9 min (Figure S1A). Since UGT78K1 has been characterised as a regiospecific 3-*O*-glycosyltransferase and the retention time of this new peak matched exactly with that of QG, it was confirmed to be QG product peak and thus was purified for further reaction. By means of a cofactor recycling system, ~3.34 g/L of QG was obtained. In this system, uridine monophosphate (UMP) is used to produce uridine triphosphate (UTP) via

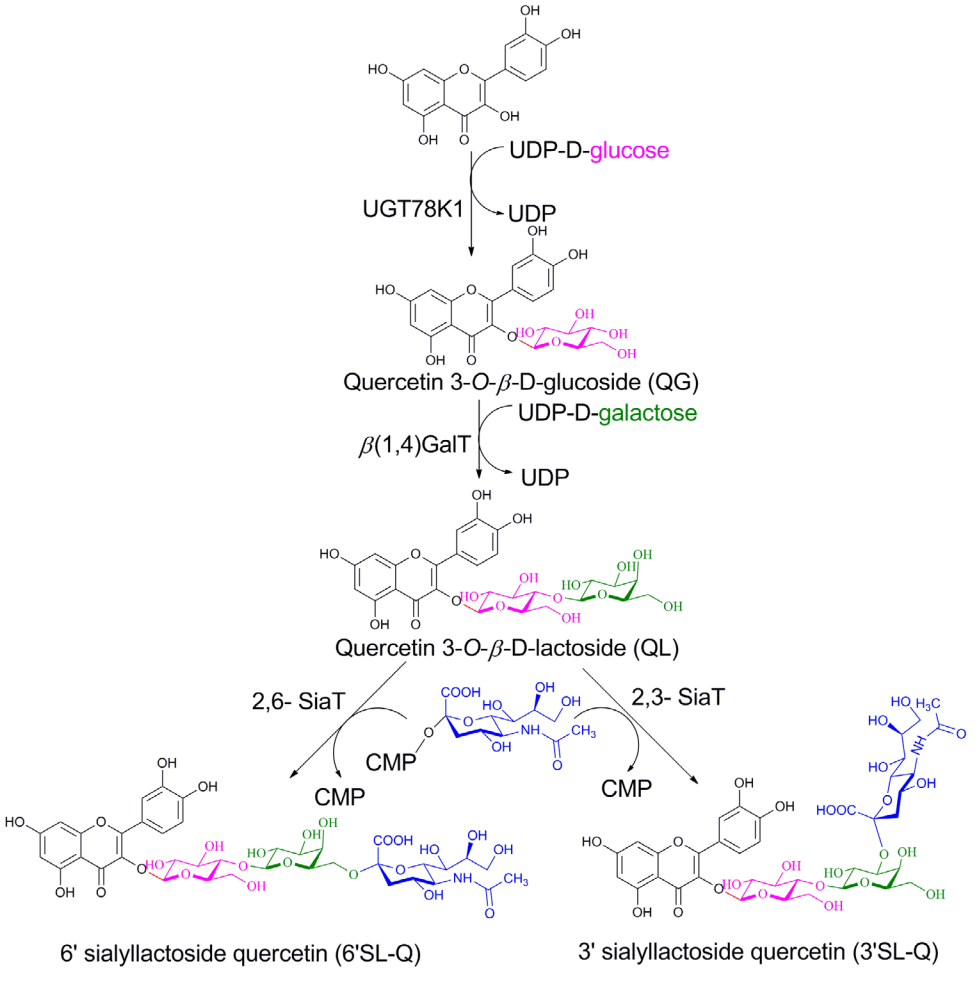

Figure 1. Schematic representation of overall strategy for enzymatic synthesis of quercetin sialoside derivatives catalyzed by glycosyltransferase (UGT78K1, β(1,4)GalT), and sialyltransferases (2,3-SiaT and 2,6-SiaT).

uridine diphosphate (UDP) with the help of enzymes UMP kinase. An adenine 5′-triphosphate (ATP) is consumed in the process and adenine 5′-diphosphate (ADP) formed is recycled by acetate kinase with the consumption of an acetyl phosphate. Glucose 1-phosphate and UTP is used for making UDP-α-D-glucose, which is used by UGT78K1 to attach D-glucose in quercetin to make QG. UDP released as a side product in the process is recycled in the system. This system was developed in our laboratory for synthesising glycosides of curcumin (Gurung et al. 2017), α-mangostin (Le et al. 2014), nargenicin A1 (Dhakal et al. 2015), and resvera-A (Shin et al. 2016) in which YjiC enzyme was used. This method uses cheap starting materials and a desired glycosylated natural product can be efficiently made with the help of regiospecific and stereospecific glycosyltransferase. The pH of the reaction was maintained whenever a decrease in pH was observed for the effective running of the reaction.

2.2. In vitro *enzyme-catalyzed quercetin-3-O-β-D-lactoside synthesis*

Similarly, the *β*-1,4-galactosyltransferase enzyme from *Helicobacter pylori* catalysed the reaction mixture of UDP-*α*-D-galactose as a sugar donor, and QG as the acceptor substrate. When analysing the sample of this reaction mixture in HPLC-PDA, a peak was observed earlier than the substrate peak at retention time of 8.3 min, while the substrate peak was at 8.6 min with more than 95% conversion [Figure S1B(iii)]. After further analysing this peak by high-resolution quadruple time-of-flight electrospray ionisation-mass spectrometry (HR-QTOF-ESI/MS) in positive ion mode, the mass spectrum was found to be *m/z* 627.1568, which resembled with that of quercetin conjugated with lactose i.e. $[QL + H]^+$, which is *m/z* 627.1561 (Figure S2A). 1.19 g/L of QL was formed in the process which was collected, purified, and further used for sialylation reaction.

2.3. In vitro *enzyme-catalysed sialyllactosyl quercetin derivatives synthesis*

Two sialyltransferases, *α*2,3-SiaT and *α*2,6-SiaT were prepared as described in experimental section and used to catalyse the independent reaction mixture containing quercetin-3-*O*-*β*-D-lactoside as acceptor substrate and CMP-*N*-acetylneuraminic acid (CMP-NeuNAc) as a sugar donor. Two sialosides of quercetin, 3′ and 6′-sialic acid conjugated products (3′SL-Q and 6′SL-Q) were produced from *α*2,3-SiaT and *α*2,6-SiaT, respectively. Each reaction mixture was first analysed by HPLC-PDA and then confirmed by LC-QTOF-ESI/MS in positive ion mode. The HPLC-PDA analyses revealed product peaks before the retention time of QL at ~ 7.2 min for 3′SL-Q, and 7.9 min for 6′SL-Q, at the conversion rate of ~95% for both products (data not shown) and 4.35 g/L of both 3′SL-Q and 6′SL-Q were produced by the reaction. The product peaks were collected and purified (Figure S1C). In the positive ion mode spectrum, mass fragments of 3′SL-Q and 6′SL-Q showed *m/z* at 918.2540 and 918.2526 corresponding to $[3'SL\text{-}Q + H]^+$ and $[6'SL\text{-}Q + H]^+$, respectively (Figs. S2B and S2C). Mass spectra of attached sugar units were clearly observed in positive ion mode (Figs. S2D and S2E). The possible structures of different mass fragmentation of 3′SL-Q and 6′SL-Q observed are presented in figures (Figure S2F).

2.4. *Compounds purification and structural elucidation*

For confirmation and determination of the structure of sialyllactose conjugated quercetin derivatives, each product was isolated by preparative-HPLC, and analysed by NMR in DMSO-d_6 (Figure S3). The ^{1}H-NMR spectrum of 3′SL-Q showed two anomeric protons with two doublets at *δ* 4.05 (*J* = 7.7 Hz) and 5.38 (*J* = 6.5 Hz), representing beta (*β*) configuration of two sugar moieties (D-glucose and D-galactose), whereas other proton signals for glucose, galactose, and sialic acid moieties were observed in the region from *δ* (1.5–5.5) ppm. The anomeric carbons of D-glucose and D-galactose were obtained at 101 and 104 ppm, respectively. The spectra for other carbons were also present in respective places as depicted in ^{13}C-NMR (Figure S3A). Similar spectra were also observed in both ^{1}H and ^{13}C-NMR analyses of 6′SL-Q. In ^{1}H-NMR, the anomeric protons of glucose and galactose appeared at δ 4.11 (d, *J* = 7.7 Hz) and 5.56 (d, *J* = 7.5 Hz), respectively. The anomeric spectra appeared exactly at the same chemical shift in both sialosides. These data confirmed conjugation of D-glucose and D-galactose in beta configuration. The ^{1}H-NMR spectra for remaining protons were also

present in the region of 3.0–5.0 ppm. Similarly, in ^{13}C-NMR analysis, the anomeric carbons of D-glucose and D-galactose appeared at δ 101 ppm and 104 ppm, respectively. Moreover, the carbon spectra of both D-glucose and D-galactose were between 60 and 80 ppm (Figure S3). Because the CMP-NeuNAc (CMP-sialic acid) does not have an anomeric proton, we confirmed its conjugation by the presence of specific groups on them. The sialic acid contains $NHCOCH_3$ moiety and CH_2 at C-3‴, which were distinct at δ (1.87 and 1.70) ppm and δ (1.88 and 1.67) ppm in 3′SL-Q and 6′SL-Q, respectively. Similarly, while analysing ^{13}C NMR of both compounds, $\underline{C}OCH_3$ was seen at δ 172.1 and 172.18 ppm, and CH_3 group was distinct at δ 23.15 and 23.16 ppm in 3′SL-Q and 6′SL-Q respectively (Table S1; Figs. S3A and S3B). These data confirmed the enzymatic synthesis of derivatives of quercetin conjugated with 3′-sialyllactose and 6′-sialyllactose. The spectra for other carbons and hydrogen were also present in respective places as shown in Table S1 and Figs. S3. In the previous studies similar groups were confirmed to be conjugated in vancomycin and epothilone A using same glycosyltransferase and sialyltransferase enzymes (Oh et al. 2011; Parajuli et al. 2016).

2.5. Biological activity

The assessment of cytotoxicity effect of Q, QG, QL, 3′SL-Q and 6′SL-Q in *in vitro* indicated that different quercetin derivatives inhibited the growth of various cancer cells with different sensitivity (Figure S4). The inhibitory effects of 3′SL-Q, 6′SL-Q, and QL were better than QG among all the tested cancer cells. Among all the four quercetin derivatives, quercetin 3-*O*-lactoside (QL) exhibited the greatest anticancer activity. Although the synthesized compounds could not show much improvement in anti-cancer activities over gastric cancer cells (AGS), liver cancer cells (HepG2) and uterine cervical cancer cells (HeLa) in compared to the aglycone, the anticancer activity was still conserved in all of them. The results were analysed using taxol treated cells as positive control against AGS, HepG2 and Hela cells (Figure S4). All the compounds tested showed very less or no cytotoxicity at 6.25 µM which is 125-fold higher than that of the highest concentration of taxol used (0.05 µM). Since taxol is used as drug to treat various cancers, it is effective to inhibit cancer cells growth at relatively low concentration ranging from 0.005 µM to 0.05 µM (Yeung et al. 1999). It exhibits complete cell cytotoxicity at the concentration between 6.25 and 200 µM at which other quercetin glycoside derivatives were tested. The inhibitory concentration (IC_{50}) value of all of the tested quercetin derivatives against HeLa cells was above 200 µM. Similarly, it was above 200 µM in the case of 6′SL-Q, 3′SL-Q and QG against HepG2 cells. But the values were 197.14 µM and 70.84 µM with QL and Q, respectively. However, IC_{50} values were lower than 200 µM in most of the derivatives against AGS cells except QG. The activity of quercetin derivative was significantly improved with the addition of a disaccharide sugar (lactose) moiety when comparing with the addition of monosaccharide sugar (glucose) in case of all the cancer cells tested.

3. Experimental

3.1. Chemicals and reagents

Standard UDP-α-D-glucose, D-glucose-1-phosphate, UDP-α-D-galactose, isopropyl-β-D-thiogalactopyranoside (IPTG), adenine 5′-triphosphate (ATP), uridine monophosphate (UMP),

and acetyl phosphate were purchased from GeneChem (Daejeon, Korea). High-performance liquid chromatography (HPLC)-grade acetonitrile and water were purchased from Mallinckrodt Baker (Phillipsburg, NJ, USA). Quercetin (Q), standard quercetin 3-*O*-*β*-D-glucoside (QG) (CAS Number 482-35-9), taxol (paclitaxel), dimethyl sulfoxide-d_6 (DMSO-d_6), and cytidine monophosphate (CMP)-*N*-acetylneuraminic acid (CMP-NeuNAc) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All the other chemicals used were of high analytical grade and were commercially available.

3.2. Plasmids, protein expression and culture condition

Escherichia coli BL21 (DE3) (Stratagene, USA) was used as the host for the expression of proteins acetate kinase (ACK-pET24ma), UMP kinase (UMK-pET15b), UDP-*α*-D-glucose synthase (GalU-pET24ma), glycosyltransferase (UGT78K1-pET28a), and *β*-1,4-galactosyltransferase [*β*(1,4)GalT -pET24a], as in the previous researches (Malla et al. 2013; Le et al. 2014; Oh et al. 2011). Likewise, previously cloned *α*2,3-SiaT in pET32a and *α*2,6-SiaT in pET15b (Oh et al. 2011) in *E. coli* (DE3) were used for protein production. All the recombinant strains were grown using respective seed culture with the supplementation of antibiotic (50 μμg/mL kanamycin or 100 μg/mL ampicillin) in Luria–Bertani (LB) media, and allowed to grow at 37 °C, until the optical density at 600 nm (OD_{600}) reached ~0.8. After that, the final concentration of 0.5 mM of IPTG was added for induction, and the culture was further allowed to grow at 20 °C for 20 h. The cells were harvested by centrifugation at 842 × *g* at 4 °C and washed with 50 mM Tris-HCl buffer of pH 7.5. The cells were sonicated, and the clear lysate was collected by high-speed centrifugation at 13,475 × *g* for 30 min at 4 °C. The clear lysate thus obtained was used for *in vitro* reactions.

3.3. Enzymatic synthesis of quercetin derivatives

3.3.1. Synthesis of quercetin 3-O-β-D-glucoside

The one-pot reaction mixture for the synthesis of quercetin 3-*O*-*β*-D-glucosides (QG) contained 100 mM Tris buffer, 20 mM $MgCl_2$, 150 mM acetyl phosphate, 50 mM glucose 1-phosphate, 1 mM ATP, 2 mM UMP, 8 mM quercetin, and ~50 μg/mL of each crude enzyme (UMK, ACK, GalU, and UGT78K1). The reactions were incubated at 37 °C for an appropriate time, and 10 μL reaction sample was diluted in 500 μL methanol at different times, to check for conversion of the product.

3.3.2. Synthesis of quercetin 3-O-β-D-lactoside

Quercetin 3-*O*-*β*-D-lactoside (QL) was prepared by enzymatic reaction, where 2 mM of QG was used as a substrate in the reaction consisting of 50 mM Tris-Cl buffer (pH 7), 10 mM $MnCl_2$, 10 mM UDP-*α*-D-galactose, and 20% crude *β*(1,4)GalT. The reaction mixture was incubated at 37 °C for 2 h, and terminated by adding a double volume of methanol.

3.3.3. Synthesis of 3′ sialyllactosyl quercetin and 6′ sialyllactosyl quercetin

CMP-NeuNAc was used as sugar donors with sialyltransferase enzymes (*α*2,3-SiaT and *α*2,6-SiaT) for the synthesis of 3′SL-Q and 6′SL-Q, respectively. The reaction condition consisted of 25 mM Tris·HCl buffer (pH 7.5), 20 mM $MgCl_2 \cdot 6H_2O$, 5 mM QL dissolved in dimethyl sulfoxide (DMSO), 10 mM of CMP-NeuNAc, and ~50 μg/mL purified sialyltransferase enzymes.

The reaction mixtures were incubated at 37 °C for 24 h, and terminated by boiling for 5 min. Denatured proteins from the reaction mixtures were separated by centrifugation at 13,475 × *g* for 30 min.

3.4. Analytical procedures

High-performance liquid chromatography-photodiode array (HPLC-PDA) analysis was performed using a reversed phase column (Mightysil RP-18 GP 250-4.6 (5 µm)) at a UV absorbance of 312 nm. The binary mobile phases were composed of solvent A (0.025% trifluoro acetic acid (TFA) in HPLC-grade water) and solvent B (100% acetonitrile, ACN). The total flow rate was maintained at 1 mL/min for the 25-min program. The program used was as follows; ACN 10% (0 min), 30% (0–5 min), 50% (5–10 min), 90% (10–15 min), 70% (15–18 min), and 10% (18–25 min). The high-resolution quadruple time-of-flight electrospray ionisation-mass spectrometry (HR-QTOF ESI/MS) analysis was performed in positive ion mode using an ACQUITY (UPLC, Waters Corp., Billerica, MA, USA) column coupled with a SYNAPT G2-S (Water Corp.) column. The purification of compounds was performed by preparative HPLC equipped with a C_{18} column (YMC-Pack ODS-AQ (250 × 20 mm I.D., 10 µm) connected to a UV detector (312 nm) using a 40-min binary program with 100% ACN and water in following condition: 20% (0–5 min), 40% (5–15 min), 90% (15–30 min), and 20% (30–40 min) at a flow rate of 10 mL/min. The purified products were dried, lyophilised, dissolved in DMSO-d_6, and subjected to 700 MHz Bruker, BioSpin nuclear magnetic resonance (NMR) analysis, for one-dimensional ^{1}H NMR and ^{13}C NMR (176 MHz).

3.5. Biological activity

The effect of Q, QG, QL, 3′SL-Q and 6′SL-Q on the proliferation and viability of different cancer cell lines were evaluated. Taxol was used as a positive control for all the cancer cell lines. AGS gastric carcinoma, HepG2 liver carcinoma, and HeLa cervical carcinoma cells were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen). All cells were maintained at 37 °C in a humidified 5% CO_2 incubator. For cell growth assay, the cells seeded at 2000 cells/well in 96-well plates (SPL Life Sciences, Korea) were treated with each compound at various concentrations for 72 h. Cell growth was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay.

4. Conclusion

In conclusion, the present study successfully synthesised two novel sialyllactose conjugated derivatives of quercetin. The structures of those derivatives were elucidated by various chromatographic and spectroscopic analyses. These new molecules were tested for their ability to inhibit various cancer cells. Although they did not show much anticancer activity, they may offer as potential compounds for other biological studies in future research.

Disclosure statement

No potential conflict of interest was reported by the authors.

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